

Kombucha Fermentation and Its Antimicrobial Activity

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Kombucha was prepared in a tea broth (0.5% w/v) supplemented with sucrose (10% w/v) by using a commercially available starter culture. The pH decreased steadily from 5 to 2.5 during the fermentation while the weight of the “tea fungus” and the OD of the tea broth increased through 4 days of the fermentation and remained fairly constant thereafter. The counts of acetic acid-producing bacteria and yeasts in the broth increased up to 4 days of fermentation and decreased afterward. The antimicrobial activity of Kombucha was investigated against a number of pathogenic microorganisms. *Staphylococcus aureus*, *Shigella sonnei*, *Escherichia coli*, *Aeromonas hydrophila*, *Yersinia enterocolitica*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Staphylococcus epidermidis*, *Campylobacter jejuni*, *Salmonella enteritidis*, *Salmonella typhimurium*, *Bacillus cereus*, *Helicobacter pylori*, and *Listeria monocytogenes* were found to be sensitive to Kombucha. According to the literature on Kombucha, acetic acid is considered to be responsible for the inhibitory effect toward a number of microbes tested, and this is also valid in the present study. However, in this study, Kombucha proved to exert antimicrobial activities against *E. coli*, *Sh. sonnei*, *Sal. typhimurium*, *Sal. enteritidis*, and *Cm. jejuni*, even at neutral pH and after thermal denaturation. This finding suggests the presence of antimicrobial compounds other than acetic acid and large proteins in Kombucha.

Keywords: *Fermented tea; Kombucha; food fermentation; antimicrobial activity; pathogenic microorganisms*

INTRODUCTION

Kombucha is a popular beverage among many traditional fermented foods across the world. It originated in northeast China (Manchuria) and later spread to Russia and the rest of the world. Kombucha is also frequently called “tea fungus” in the literature, although there is actually no fungus involved in the fermentation (Benk, 1988; Reiss, 1987, 1994; Steinkraus et al., 1996; Liu et al., 1996; Sievers et al., 1995). This beverage reportedly exerts a number of medicinal effects, for example, against metabolic disease, arthritis, psoriasis, constipation, indigestion, and hypertension, but there is no solid scientific evidence available yet for its efficacy (O'Neill, 1994; Jacobs, 1995). By virtue of the numerous health-promoting aspects reported and the easy and safe preparation of this beverage at home, it has gained popularity as other traditional beverages. Kombucha is a symbiotic growth of bacteria (*Acetobacter xylinum*, *Acetobacter xylinoides*, *Bacterium gluconicum*) and yeast strains (*Schizosaccharomyces pombe*, *Saccharomyces ludwigii*, *Saccharomyces cerevisiae*, etc.) cultured in a sugared tea (Hermann, 1928a,b; Reiss, 1987; Herrera and Calderon-Villagomez, 1989). The exact microbiological composition also depends on the source of inoculums of the tea fermentation. Growth patterns of these microorganisms during the fermentation process of Kombucha are not well documented. Cellulose produced during the fermentation by *Ac. xylinum* appears as a thin film on top of the tea where the cell mass of bacteria and yeasts is attached. This fungus-like mixture of

microorganisms and cellulose is likely why Kombucha is also called “tea fungus” (the term indicates such a complex in the text hereafter). Glucose liberated from sucrose is metabolized for the synthesis of cellulose and gluconic acid by *Acetobacter* strains. Fructose is metabolized into ethanol and carbon dioxide by yeasts. Ethanol is oxidized to acetic acid by *Acetobacter* strains. Organic acids produced during fermentation shield the symbiotic colony from contamination with unwanted foreign microorganisms that are not part of the tea fungus (Blanc, 1996; Greenwalt et al., 1998). An optimum fermentation time is required for the production of a drinkable Kombucha. Longer fermentation often results in the production of too high levels of acids that may pose potential risks when consumed. Recent research on Kombucha has proved that its antimicrobial activity against pathogenic microorganisms is largely attributable to acetic acid (Steinkraus et al., 1994; Greenwalt et al., 1998). Acetic acid is known to inhibit and destroy a number of Gram-positive and Gram-negative microorganisms (Levine and Fellers, 1940). Numerous reports on unfermented tea extracts at higher concentrations as well as pure tannins suggest a potential antibiotic effect against a number of pathogenic microorganisms (Toda et al., 1989a,b, 1991; Diker et al., 1991, 1994). The physiological changes that occur during the fermentation process of Kombucha and the possible relationship of these changes with speculative curative and antimicrobial effects are not yet clear and need further systematic investigations.

In this paper we report the microbial and chemical changes during Kombucha fermentation and its anti-

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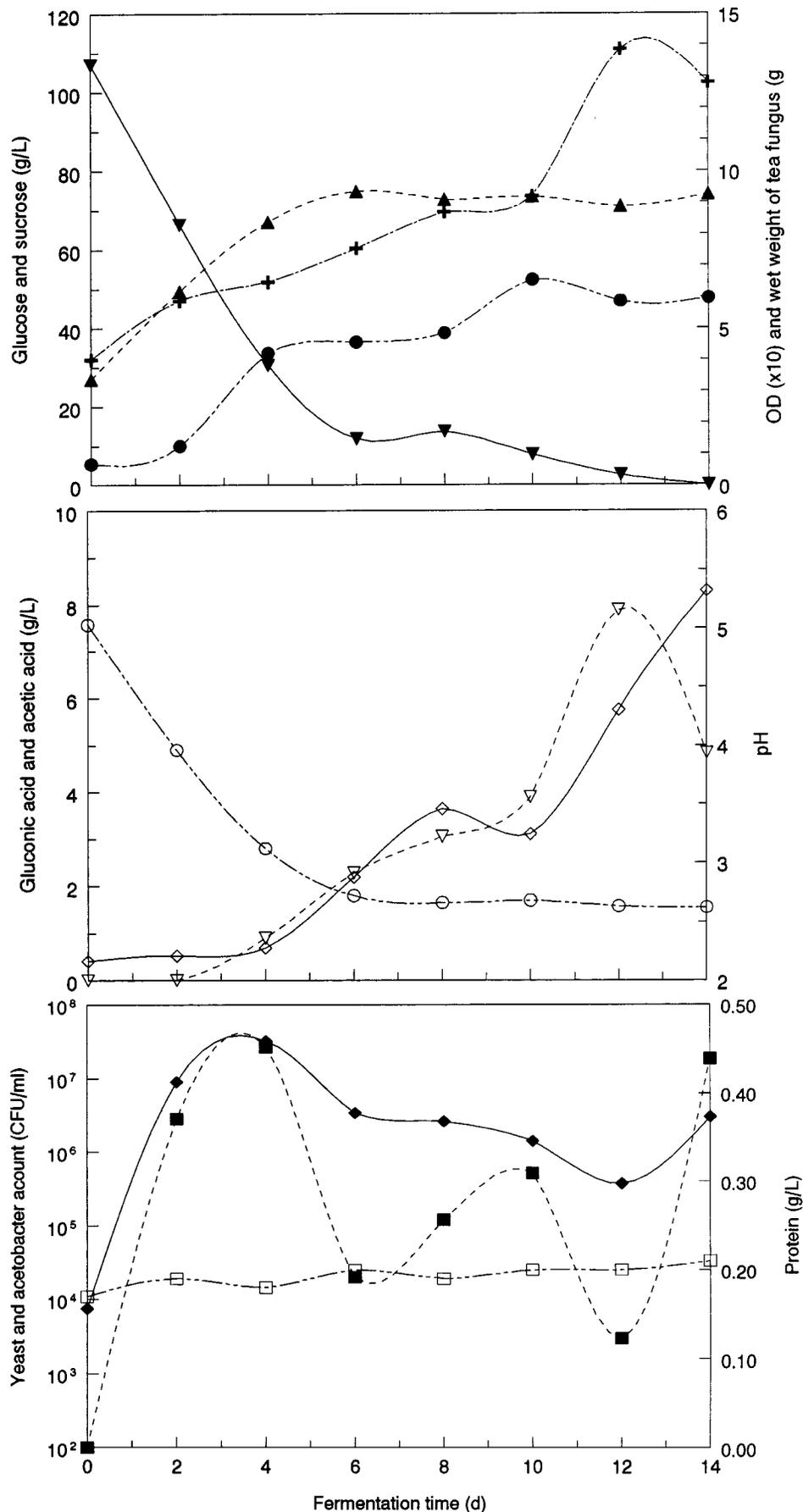


Figure 1. Analysis results during the course of Kombucha fermentation: (▲) glucose; (▼) sucrose; (●) OD at 600 nm; (+) wet weight of tea fungus; (▽) gluconic acid; (◇) acetic acid; (○) pH; (■) yeast count; (◆) acetic acid-producing bacteria count; (□) protein.

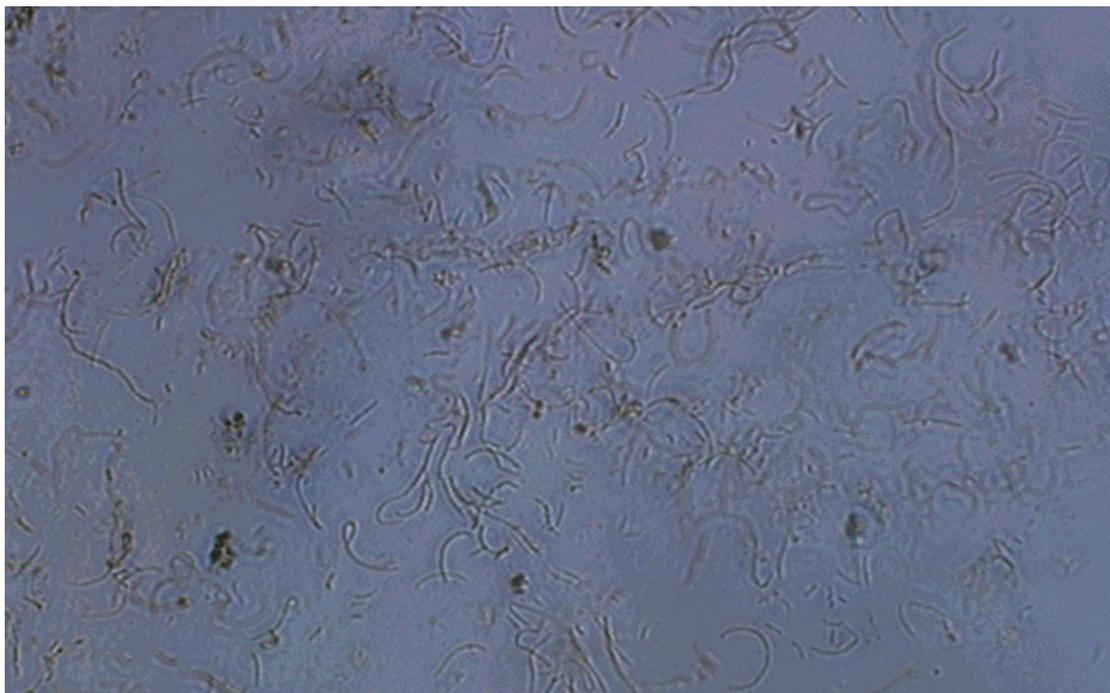


Figure 2. Phase contrast micrograph of tea fungus after hydrolysis of cellulose (bacteria and yeasts are indicated).

microbial activity against a broad spectrum of Gram-negative and Gram-positive pathogenic microorganisms.

MATERIALS AND METHODS

Starter Cultures. Starter cultures, or commercial Kombucha, were purchased from a local pharmacy in Utrecht, The Netherlands. The product is marketed by Pharma Import, Beverlo-Berigen, Belgium.

Kombucha Preparation. Sucrose (10% w/v) and glucose (2.5% w/v) were added to demineralized water that had been just boiling for 15 min. Subsequently, black tea (C'estbon, Lapsang souchon, 0.5% w/v) was added and allowed to steep for 15 min and then filtered through a sterile sieve. The tea was then cooled to 25 °C, and 400 mL of tea was aliquoted into a 750 mL glass bottle that had been previously sterilized at 121 °C for 20 min. The tea broth was inoculated with 5 g of freshly grown tea fungus that had been cultured in the same medium for 14 days, and the bottle was covered with sterile tissue paper towels to allow aeration. Fermentation was carried out in a dark incubator at 25 °C. Sampling was done by taking one bottle from the incubator at two-day intervals. Samples were used for the measurement of microbial and chemical changes.

Determination of pH. The pH of the samples was measured with an electronic pH meter (PHM 82, Standard pH Meter, Radiometer Copenhagen).

Monitoring of Microbial Growth. The weight of the wet tea fungus and OD of the fermentation broth at 600 nm were measured throughout the fermentation. Wet weight of tea fungus was measured by draining the tea fungus on a filter paper under vacuum conditions until no free water was drained out. Both wet weight and OD were used as indicators of microbial growth.

Protein Estimation. Protein content in the fermentation broth was determined according to the method described by Bradford (1976) by using a Cobas Mira Plus autoanalyzer (Roche, Basel, Switzerland).

Microbiological Analysis. For monitoring the growth of acetic acid-producing bacteria, liquid tea samples were plated on WL nutrient agar (CM309, Oxoid) containing 4 mg of cycloheximide (C7698, Sigma) per liter to prevent yeast growth. For total yeast counting, the same samples were plated on OGYA medium (oxytetracycline glucose-yeast extract agar, CM545, Oxoid) containing oxytetracycline (SR73, Oxoid).

Plates were incubated at 30 °C for acetic acid-producing bacteria and at 25 °C for yeasts. Tea samples were also analyzed for the presence of bacteria other than acetic acid-producing bacteria by plating samples on TSA medium (tryptone soy agar, CM131, Oxoid) plates containing 1 g/L delfocid (DSM, Delft, The Netherlands) for inhibiting mold growth.

Target Strains and Cultivation Conditions. *Staphylococcus aureus* (ATCC 6538), *Shigella sonnei* (ATCC 29930), *Escherichia coli* (ATCC 8739), *Aeromonas hydrophila* (ATCC 35654), *Yersinia enterocolitica* (ATCC 9610), *Pseudomonas aeruginosa* (ATCC 15442), *Enterobacter cloacae* (ATCC 13047), *Staphylococcus epidermidis* (ATCC 12228), *Salmonella enteritidis* (TNO collection, B308), *Salmonella typhimurium* (ATCC 13311), *Bacillus cereus* (ATCC 9139), and *Listeria monocytogenes* (ATCC 19114) were grown and maintained in TSB medium (tryptone soy broth, CM129, Oxoid). *Zygosaccharomyces bailii* (TNO collection, Y394) and *Candida albicans* (ATCC 10231) were cultured on OGYA medium. *Helicobacter pylori* (ATCC 43504 D) and *Campylobacter jejuni* (NTCC 11351) were grown under microaerophilic condition and maintained on HIBA (heart infusion broth, Difco) containing 5% (v/v) sheep blood.

Hydrolysis of Cellulose in Tea Fungus. Cellulose in the tea fungus was hydrolyzed with commercial cellulase (Sigma) in an acetate buffer (pH 5.5) at 50 °C for 1 h, as described by the enzyme manufacturer.

Antimicrobial Activity. Antimicrobial activity was demonstrated by agar diffusion assay. TSA medium (20 mL) was poured into each Petri dish (90 mm diameter). Suspensions (100 μ L) of target strain cultured for 24 h were spread on the plates uniformly, and wells of 9 mm diameter were made with a sterile metal tube by means of a vacuum pump. Kombucha samples were centrifuged at 40000g force (Du Pont centrifuge, Sorvall RC-5B) for 15 min to remove cell debris. Sterile supernatant was obtained by filtering the supernatant through a sterile microfilter (Millex-GV filter, 0.22 μ m pore size, Millipore). Sterile samples (100 μ L) were then transferred into the wells of agar plates inoculated with target strains. The plates were first put at 4 °C for 2 h to make a prediffusion of tea sample into the agar. The plates were then incubated at 37 °C. The diameter of the inhibition zone was measured after 12–15 h.

For the purpose of control and comparison, acetic acid samples at the same concentration as that of fermented tea after 14 days (8.5 g/L) were prepared and sterile filtered for

Table 1. Antimicrobial Effect of Kombucha^a

| target microorganism | testing materials | | | | | | | | | | | |
|---|--------------------------------------|------------------------------|--------|---------------------------------------|--------|--------|--------|--------|---------|---------|---------|-------|
| | acetic acid ^d (pH 2.3) | unfermented tea ^b | | fermented tea (Kombucha) ^c | | | | | | | | |
| | | pH 5.0 | pH 7.0 | 0 days | 2 days | 4 days | 6 days | 8 days | 10 days | 12 days | 14 days | |
| <i>En. cloaceae</i> (ATCC 13047) | +++ | - | - | natural pH ^e | - | - | - | - | - | + | + | ++ |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>P. aeruginosa</i> (ATCC 15442) | +++ | - | - | natural pH | - | - | - | - | - | - | - | ++ |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>B. cereus</i> (ATCC 9139) | + | - | - | natural pH | - | - | - | - | - | + | + | + |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>E. coli</i> (ATCC 8739) | ++ | - | - | natural pH | - | ++ | +++ | +++ | +++ | ++++ | +++ | ++++ |
| | | | | pH 7.0 | - | ++ | ++ | +++ | +++ | ++++ | +++ | +++ |
| | | | | heat-denatured | - | ++ | +++ | +++ | +++ | +++ | +++ | +++ |
| <i>A. hydrophila</i> (ATCC 35654) | ++ | - | - | natural pH | - | + | + | + | + | + | ++ | ++ |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>S. typhimurium</i> (ATCC 13311) | +++ | - | - | natural pH | - | +++ | ++++ | ++++ | ++++ | +++++ | +++++ | +++++ |
| | | | | pH 7.0 | - | +++ | ++++ | ++++ | ++++ | +++++ | +++++ | +++++ |
| | | | | heat-denatured | - | +++ | ++++ | ++++ | ++++ | +++++ | +++++ | +++++ |
| <i>S. enteritidis</i> | ++ | - | - | natural pH | - | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | | | | pH 7.0 | - | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | | | | heat-denatured | - | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| <i>St. epidermis</i> (ATCC 12228) | + | - | - | natural pH | - | - | - | - | - | + | + | + |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>L. monocytogenes</i> (ATCC 19114) | ++ | - | - | natural pH | -? | + | + | + | ++ | ++ | ++ | ++ |
| | | | | pH 7.0 | -? | + | + | + | + | + | + | + |
| | | | | heat-denatured | -? | + | + | + | + | + | + | + |
| <i>Y. enterocolitica</i> (ATCC 9610) | ++ | - | - | natural pH | - | - | - | - | + | + | + | + |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>St. aureus</i> (ATCC 6538) | ++ | - | - | natural pH | - | - | - | + | + | + | + | ++ |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>Sh. sonnei</i> (ATCC 29930) | ++ | - | - | natural pH | - | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | | | | pH 7.0 | - | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| | | | | heat-denatured | - | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ |
| <i>Cm. jejuni</i> | ++++ | +++ | +++ | natural pH | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | | | | pH 7.0 | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| | | | | heat-denatured | +++ | +++ | +++ | +++ | +++ | +++ | +++ | +++ |
| <i>H. pylori</i> (ATCC 43504 D) | +++++ | - | - | natural pH | - | ++ | ++ | +++ | +++ | +++ | ++++ | ++++ |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |
| <i>Cn. albicans</i> (ATCC 10231) | - | - | - | natural pH | - | - | - | + | ++ | ++ | ++ | ++ |
| | | | | pH 7.0 | - | - | - | + | + | + | + | + |
| | | | | heat-denatured | - | - | - | + | + | + | + | + |
| <i>Z. bailii</i> | - | - | - | natural pH | - | - | - | - | - | - | - | - |
| | | | | pH 7.0 | - | - | - | - | - | - | - | - |
| | | | | heat-denatured | - | - | - | - | - | - | - | - |

^a Diameter of halo zone: -, no inhibition; +, 10–15 mm; ++, 15–20 mm; +++, 20–25 mm; +++++, 25–30 mm; ++++++, 30–35 mm.

^b Unfermented tea samples were prepared the same as that for making Kombucha without adding any sugar, and 1 M HCl or 1 M NaOH was used to adjust their pH. ^c Heat-denatured fermented tea samples were treated at 80 °C for 30 min, whereas pH 7 fermented tea samples were adjusted with 1 M NaOH. ^d Acetic acid samples were prepared according to the acetic acid concentration of Kombucha, 8.5%, and sterile filtered. ^e Natural pH refers to the pH value of the sample without any adjustment. Heat-denatured samples were treated at 80 °C for 30 min.

antimicrobial test as described above for fermented tea samples. In the same way, pH 5 and 7 samples of unfermented and fermented tea were obtained by adjusting the pH with 1 M HCl or 1 M NaOH. Heat-denatured samples were treated at 80 °C for 30 min. They were then sterile filtered and tested for their antimicrobial activity in the same way as described above.

RESULTS AND DISCUSSION

Microbial and Chemical Changes during Kombucha Fermentation. The microorganisms utilized the carbon source and started producing cellulose, which appeared as a thin layer on top of the broth. The wet weight of the tea fungus and the OD of the tea broth were found to increase with fermentation time, as

shown in Figure 1. Microscopic evaluation revealed that the tea fungus mainly contains yeasts, bacteria, and cellulose produced during the fermentation process. The pH of the tea broth decreased with fermentation time. During the fermentation process, yeasts and bacteria metabolize sucrose into a number of organic acids, such as acetic acid and gluconic acid. Due to an increased concentration of these organic acids, the pH decreased from 5 to 2.5 within 6 days of fermentation and remained stable thereafter. These observations are in agreement with the findings of other studies (Steinkraus et al., 1994; Greenwalt et al., 1998). Although no other nitrogen source was added to the tea before fermentation, the protein level increased slightly with fermentation time. These proteins likely represent extracellular

proteins secreted by yeasts and bacteria during the fermentation process or originally existing in the tea broth.

Microbiology of Kombucha. The tea fungus represents a symbiotic growth of acetic acid bacteria and yeasts attached to cellulose. However, the microbiological growth pattern of these microorganisms during fermentation has not yet been studied in detail. This is probably due to difficulties in separating the cell mass from cellulose in the tea fungus complex. We studied the growth patterns of acetic acid bacteria and yeasts in the tea broth as a function of fermentation time. The results of the plate agar count method for analysis of acetic acid-producing bacteria and yeasts in tea samples are shown in Figure 1. *Acetobacter* and *Gluconobacter* counts were tried by plating tea samples on agar plates containing cycloheximide, which inhibits the growth of yeasts. However, it was not possible to distinguish *Acetobacter* and *Gluconobacter* strains on agar plates and, therefore, bacterial strains were expressed as acetic acid-producing bacteria. The acetic acid-producing bacterial count increased rapidly through 4 days of fermentation, declined rapidly by 6 days of fermentation, and thereafter continued to decrease (Figure 1). The decreased number of acetic acid bacteria after 4 days of fermentation was likely caused by acid shock (low pH), which influenced the multiplication of bacteria (Figure 1). A slight secondary growth was observed after 12 days of fermentation, likely due to multiplication of acid-tolerant bacterial strains. Yeast counts followed a trend very similar to that shown by acetic acid bacteria. The total yeast count increased rapidly until 4 days of fermentation and declined drastically by 6 days of fermentation (Figure 1). A secondary growth of yeasts was observed after 12 days of fermentation. No bacterial growth was observed in tea samples plated on TSA-containing delfocid. This finding suggests that, except for acetic acid-producing bacteria, other bacteria can hardly grow in Kombucha. In addition, this also excludes the possible existence of known antibiotics in the broth produced by *Bacillus* or *Streptomyces* species.

Tea fungus was hydrolyzed with commercial cellulase to investigate the association of bacteria and yeast with cellulose. Complete hydrolysis of cellulose was achieved by applying cellulase at 1 unit of cellulase/mg of tea fungus. Phase contrast microscopic observations of hydrolyzed tea fungus revealed the presence of acetic acid bacteria and yeasts in the tea fungus (Figure 2). A similar association of *Acetobacter* and yeast strains has been noted by Reiss (1994). The association of bacteria and yeasts with tea fungus serves normally as a starter culture for new Kombucha. In the literature, acetic acid bacteria in Kombucha have been identified as *Acetobacter* sp., NRRL B-2357 (Hesseltine, 1965), and *Ac. xylinum* (Mayser et al., 1995; Sievers et al., 1995). Among the yeast strains, *Pichia* sp., *Cn. albicans*, *Z. rouxii*, *Zygosaccharomyces* sp., *Brettanomyces* sp., and *Saccharomyces* sp. have been identified (Hesseltine, 1965; Kozaki et al., 1972; Mayser et al., 1995). Obviously, the microbiological composition of tea fungus largely depends on its source, which may influence specific characteristics of the product, Kombucha.

Antimicrobial Activity of Kombucha. The antimicrobial activity of Kombucha under different conditions against a number of pathogenic microorganisms is presented in Table 1. Unfermented tea and acetic acid were used as controls. Fermented samples were tested

either at their natural pH (acidic, as shown in Figure 1) or adjusted to neutral pH (7.0). Heat-treated samples were tested to check whether the active components are thermostable, to confirm whether the active components are large proteins. Unfermented tea samples had hardly any antimicrobial activity against target microorganisms except for *Cm. jejuni*. Acetic acid was inhibitory toward all of the bacteria, but not the yeasts. In fact, acetic acid showed the same inhibition as Kombucha toward 10 of the 14 bacteria. In the other four cases (*E. coli*, *Sal. enteritidis*, *Sal. typhimurium*, and *Sh. sonnei*), Kombucha had its strongest antimicrobial effects, and these were also exhibited at pH 7.0 and after heating. Furthermore, although acetic acid had no inhibitory effect on yeast, Kombucha did inhibit the growth of *Cn. albicans*. This implies the existence of an antimicrobial component other than acetic acid and large proteins.

There are numerous reports that the polyphenols/tannins extracted from tea inhibit a broad spectrum of Gram-positive and Gram-negative bacteria. Among the catechins tested, epigallocatechin, epicatechin gallate, and epigallocatechin gallate have been found to be inhibitory for the growth of *S. aureus* and *V. cholerae* (Toda et al., 1991). Diker et al. (1991, 1994) reported that the extracts of green and black tea can inhibit *Cm. jejuni*, *E. coli*, and *H. pylori*. Recently, Greenwalt et al. (1998) have tested the antimicrobial activity of Kombucha as well as normal tea extracts prepared at different concentrations and found that the inhibitory effects of Kombucha increased with the tea concentration. In our studies, the concentration of tea broth was 0.5% for the preparation of Kombucha. The polyphenol/tannin level in such a low concentration of tea was unlikely to have an inhibitory effect against the target microorganisms, as shown in Table 1 as the unfermented tea. The only exception is *Cm. jejuni*. Hence, these findings suggest the presence of an antimicrobial compound other than acetic acid, large proteins, and catechins in Kombucha.

Antimicrobial activity increased with fermentation time, as seen in almost all cases tested except for *Cm. jejuni* and *Z. bailii*. This also implies that the active antimicrobial components are very likely metabolites produced by the bacteria and/or yeasts responsible for the fermentation of Kombucha.

At present a characterization of antimicrobial compounds is in progress.

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